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बौद्धिक सम्पदा भारत
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THE PATENTS ACT, 1970

IT IS HEREBY CERTIFIED THAT, the annex is a true copy of the Patent Application and Provisional Specification filed on **25/04/2005** in respect of Patent Application No. **505/MUM/2005** of **Cadila Pharmaceuticals Ltd.**, "Cadila Corporate Campus", Sarkhej-Dholka Road, Bhat, Ahmedabad, 382210, Gujarat, India.

This certificate is issued under the powers vested in me under Section 147 (1) of the Patents Act, 1970.

Dated this 12th day of September 2008.

(A.T. PATRE)
ASSTT. CONTROLLER OF PATENTS & DESIGNS

14644
505/MUM/05

FORM 1
THE PATENTS ACT, 1970
APPLICATION FOR GRANT OF A PATENT
(See section 5(52), 7, 54, AND 135 and Rule 33A)

1. We Cadila Pharmaceuticals Ltd., "Cadila Corporate Campus", Sarkhej-Dholka Road, Bhat, Ahmedabad - 382210, Gujarat, India, an Indian Company.

2. Hereby declare:
   a. That we are in possession of an invention for the **VACCINE ADJUVANTS** That the Provisional Specification relating to the invention is filed with this application.
   b. That there is no lawful ground of objection to the grant of patent to us.

3. Further declare that the inventors for the said invention are

   a. Mr. Indravadan Ambalal Modi, Cadila Pharmaceuticals Ltd., "Cadila Corporate Campus", Sarkhej-Dholka Road, Bhat, Ahmedabad - 382210, Gujarat, India, Nationality, Indian.
   b. Dr.Rajiv Indravadan Modi, Cadila Pharmaceuticals Ltd., "Cadila Corporate Campus", Sarkhej-Dholka Road, Bhat, Ahmedabad - 382210, Gujarat, India, Nationality, Indian.
   c. Dr.Prasanta kumar Ghosh, Cadila Pharmaceuticals Ltd., "Cadila Corporate Campus", Sarkhej-Dholka Road, Bhat, Ahmedabad - 382210, Gujarat, India, Nationality, Indian.
   d. Mr.Nirav Desai, Cadila Pharmaceuticals Ltd., "Cadila Corporate Campus", Sarkhej-Dholka Road, Bhat, Ahmedabad - 382210, Gujarat, India, Nationality, Indian.

   e. Dr.. Bakulesh Mafatlal Khamar, Cadila Pharmaceuticals Ltd., "Cadila Corporate Campus", Sarkhej-Dholka Road, Bhat, Ahmedabad - 382210, Gujarat, India, Nationality, Indian.

4. That we are the assignee of the true and first inventors

5. That our address for service in India is as follows: Dr. Bakulesh Mafatlal Khamar, Cadila Pharmaceuticals Ltd., "Cadila Corporate Campus", Sarkhej-Dholka Road, Bhat, Ahmedabad - 382210, Gujarat, India.

.....5000/- रुपये नकद/चेक/पेनी ऑर्डर द्वारा
CR संख्या 14644 दि. 25/4/05
के तहत प्राप्त हुए।

सहा. अधिकारी ऐकडिया

6. Following declaration was given by inventors:
We the true and first inventors for this invention declare that the applicant herein is our assignee

a. Mr. Indravadan Ambalal Modi, Cadila Pharmaceuticals Ltd., "Cadila Corporate Campus", Sarkhej-Dholka Road, Bhat, Ahmedabad - 382210, Gujarat, India, Nationality, Indian

Mr. Indravadan Ambalal Modi
Date: April 21, 2005

b. Dr.Rajiv Indravadan Modi, Cadila Pharmaceuticals Ltd., "Cadila Corporate Campus", Sarkhej-Dholka Road, Bhat, Ahmedabad - 382210, Gujarat, India, Nationality, Indian.

Dr. Rajiv Indravadan Modi
Date: April 21, 2005

c. Dr.Prasanta kumar Ghosh, Cadila Pharmaceuticals Ltd., "Cadila Corporate Campus", Sarkhej-Dholka Road, Bhat, Ahmedabad - 382210, Gujarat, India, Nationality, Indian.

Dr.Prasanta kumar Ghosh
Date: April 21, 2005

d. Mr.Nirav Desai, Cadila Pharmaceuticals Ltd., "Cadila Corporate Campus", Sarkhej-Dholka Road, Bhat, Ahmedabad - 382210, Gujarat, India, Nationality, Indian.

Mr. Nirav Desai
Date: April 21, 2005

e. Dr. Bakulesh Mafatlal Khamar, Cadila Pharmaceuticals Ltd., "Cadila Corporate Campus", Sarkhej-Dholka Road, Bhat, Ahmedabad - 382210, Gujarat, India, Nationality, Indian.

Dr. Bakulesh M Khamar
Date: April 21, 2005

7. That to the best of our knowledge, information and belief the fact and matters stated herein are correct and that there is no lawful ground of objection of the grant of patent to us in this application.
8. Following are the attachment with this application:
   (a) Provisional specification (3 copies)
   (b) Statement and Undertaking on Form 3 (3 copies)
   (c) Fees Rs. 5000/- (Rupees Five thousand only) in cheque bearing no. 814363, dated 13.04.2005 on Bank of Baroda.
9. We request that a patent may be granted to us for the said invention.

Dated, this April 21, 2005

Dr. Bakulesh M Khamar
Director - Research
FOR CADILA PHARMACEUTICALS LTD.

To,
The Controller of Patents
The Patents Office Branch, Mumbai

FORM 2

THE PATENTS ACT, 1970
(39 0F 1970)
THE PROVISIONAL SPECIFICATION
(See section 10)

1. **VACCINE ADJUVANTS**

2. **CADILA PHARMACEUTICALS LTD., "CADILA CORPORATE CAMPUS", SARKHEJ-DHOLKA ROAD, BHAT, AHMEDABAD - 382210, GUJARAT, INDIA, AN INDIAN COMPANY.**

3. **THE FOLLOWING SPECIFICATION DESCRIBES AND ASCERTAINS THE NATURE OF THIS INVENTION AND THE MANNER IN WHICH IT IS TO BE PERFORMED.**

VACCINE ADJUVANTS

FIELD OF INVENTION:

The present invention pertains to adjuvants, such as adjuvants for at least one epitope of interest or antigen (including allergen), immunological, immunogenic, antigenic or vaccine compositions comprising the adjuvants, and methods for making and using the same. More in particular, the present invention relates to Mycobacterium w or fractions thereof as adjuvants, such as adjuvants for at least one epitope of interest or antigen (including allergen), immunological, immunogenic, antigenic or vaccine compositions comprising the adjuvants, and methods for making and using the same.

Its also relate to vaccine composition containing Mycobacterium w or fractions thereof as adjuvants and process of making same.

The inventive adjuvants surprisingly favorably alter the immune response by a vertebrate, e.g., mammal, to the epitope of interest or antigen combined therewith. And, the invention pertains to compositions, uses and methods arising from this observation.

BACKGROUND OF THE INVENTION

Immunogenicity can be significantly improved if an antigen is co-administered with an adjuvant, commonly used as 0.001% to 50% solution in phosphate buffered saline. Adjuvants are substances that enhance the immune response to antigens, but are not necessarily immunogenic themselves. Adjuvants may act by retaining the antigen locally near the site of administration to produce a depot effect facilitating a slow, sustained release of antigen to cells of the immune system. Adjuvants can also attract cells of the immune system to an antigen depot and stimulate such cells to elicit immune responses.

Aluminum hydroxide and aluminum phosphate (collectively commonly referred to as alum) are routinely used as adjuvants in human and veterinary vaccines. The efficacy of alum in increasing antibody responses to diphtheria and tetanus toxoids is well established and, more recently, a HBsAg vaccine has been adjuvanted with alum.

A wide range of extrinsic adjuvants can provoke potent immune responses to antigens. These include saponins complexed to membrane protein antigens (immune stimulating complexes), pluronic polymers with mineral oil, killed mycobacteria in mineral oil, Freund's complete adjuvant, bacterial products, such as muramyl dipeptide (MDP) and lipopolysaccharide (LPS), as well as lipid A, and liposomes. To efficiently induce humoral immune response (HIR) and cell-mediated immunity (CMI), immunogens are preferably emulsified in adjuvants.

Chemically defined adjuvants, such as monophosphoryl lipid A, phospholipid conjugates have been investigated (see Goodman-Snitkoff et al., J. Immunol. 147:410-415 (1991)) as has encapsulation of the protein within a proteoliposome (see Miller et al., J. Exp. Med. 176:1739-1744 (1992)). Synthetic polymers have also been evaluated as adjuvants. These include the homo- and copolymers of lactic and glycolic acid, which have been used to produce microspheres that encapsulate antigens (see Eldridge et al., Mol. Immunol. 28:287-294 (1993)).

Nonionic block copolymers are another synthetic adjuvant being evaluated. Adjuvant effects have also been investigated for low molecular weight copolymers in oil-based emulsions and for high molecular weight copolymers in aqueous formulations (Todd et al., Vaccine 15:564-570 (1997)).

At this time however, the only adjuvant widely used in humans has been alum. Other adjuvants, such as Sponin, Quil A, and the water-in-oil adjuvant,

Freund's with killed tubercle bacilli (Freund's complete) or without bacilli (Freund's incomplete), have had limited use in humans due to their toxic effects; and, concerns have been raised as to undesirable effects in animals. Simply, many adjuvant formulations have been described but most are never accepted for routine vaccines, and few have been evaluated in humans, mainly due to their toxicity.

In fact, the adjuvant effect of most experimental adjuvants has been associated with the adverse effects they elicit.

For instance, adjuvants that act as immunostimulators such as muramyl dipeptide, lipopolysaccaride, lipid A, monophosphoryl lipid A, and cytokines such as IL-2 and IL-12 can also cause systemic side-effects (general toxicity, pyrogenicity), limiting their use.

Accordingly, a problem in the art is a need for adjuvants. There remains a need for improved adjuvants that are safe and economical to manufacture for human and veterinary vaccines (reviewed by Gupta and Siber, Vaccine 13:1263-1276 (1995)).

US patent 6,224,882 describes insect cells & its fraction as an adjuvant for immunogenic, immunological, antigenic or vaccine composition. The insect as per invention can be S.frugiperda.

US patent 6,355,414 describes acemannan polysaccharide as an adjuvant.

US patent 6,306,404 describes adjuvant & vaccine compositions of mono phosporyl lipid A, sugar and optionally an amine based surfactant.

US patent 6,231,859 describes saponin combination as an adjuvant.

US patent 6,060,068 describes interleakin-2 as an adjuvant to vaccines.

US patent 6,355,256 describes QS-21 & IL-12 as adjuvants.

US patent 6,103,697, 6,228,373 & 6,228,374 describes peptides as adjuvants.

JP 11106351, JP 9268130 & AU 780054 describes oil adjuvants.

Surprisingly it is observed that Mycobacterium w & its constituents fulfill the above requirement of improved adjuvant, which is safe & economical to manufacture.

Mycobacterium W is a rapidly growing Mycobacterium.Mycobacterium w is a non-pathogenic, cultivable, atypical mycobacterium, with biochemical properties and fast growth characteristics resembling those belonging to Runyons group IV class of Mycobacteria in its metabolic and growth properties but is not identical to those strains currently listed in this group. It is therefore thought that (M_w) is an entirely new strain. The species identity of M_w has been defined by polymerase chain reaction DNA sequence determination.

Following example describes the process of obtaining Mycobacteium W

A. Culturing of Mycobacterium w.

i) Preparation of calture medium.

Mycobacterium w is cultured on solid medium like L J medium or liquid medium like middle brook medium or sauton's liquid medium.

For better yield middle brook medium is enriched. It can be preferably enriched by addition of glucose, bactotryptone, and BSA. They are used in ratio of 20:30:2 preferably.

The enrichment medium is added to middle brook medium. It is done preferably in ratio of 15:1 to 25:1 more preperably in ratio of 20:1.

ii) Bioreactor operation

a) Preparation of vessel:

The inner contact parts of the vessel (Joints, mechanical seals, o-ring/ gasket grooves, etc.) should be properly cleaned to avoid any contamination. Fill up the vessel with 0.1 N NaOH and leave as such for 24 H to remove pyrogenic materials and other contaminants. The vessel is then cleaned first with acidified water, then wit ordinary water. Finally, the vessel is rinsed with distilled water (3 times) before preparing medium.

b) Sterilization of bioreactor

The bioreactor containing 9L distilled water is sterilized with live steam(indirect). Similarly the bioreactor is sterilized once more with Middlebrook medium. The other addition bottles, inlet/outlet air filters etc. are autoclaved (twice) at 121^{0}C for 15 minutes. Before use, these are dried at 50^0 C oven.

c) Environmental parameter

i. Temprature: 37± 0.5^0 C

ii. pH : 6.7 to 6.8 initially.

B. Harvesting and concentrating

It is typically done at the end of 6th day after culturing under aseptic condition. The concentration of cells (palletisation) is done by centrifugation.

C. Washing of cells

The pallet so obtained is washed minimum three times with normal saline. It can be washed with any other fluid which is preferably isotonic.

D. Adding pharmaceutically acceptable carrier.

Pyrogen free normal saline is added to pallet. Any other pyrogen free isotonic fluid can be used as a pharmaceutical carrier. The carrier is added in amount so as get to desired concentration of active in final form.

E. Adding preservative

To keep the product free from other contaminating bacteria for its self life preservative is added. Preferred preservative is thiomesol which is used in final concentration of 0.01 % w/v.

F. Terminal Sterilization

Terminal sterilization can done by various physical methods like application of heat or ionizing radiation or sterile filtration.

Heat can be in the form of dry heat or moist heat. It can also be in the form of boiling or pasturisation.

Ionizing radiation can be ultraviolet or gamma rays or mircrowave or any other form of ionizing radiation.

It is preferable to autoclave the final product.

This can be done before after filling in a final packaging.

G. Quality Control

i. The material is evaluated for purity, sterility.

ii. The organisms are checked for acid fastness after gram staining.

iii. Inactivation test : This is done by culturing the product on L J medium to find out any living organism.

iv. Pathogenicity and/or contamination with pathogen.

The cultured organisms are infected to Balb/c mice.
None of the mice should die and all should remain healthy and gain weight. There should not be any macroscopic or microscopic lesions seen in liver, lung spleen or any other organs when animals are killed upto 8 weeks following treatment.

v. Biochemical Test:

The organism is subjected to following biochemical tests:

a) Urease

b) Tween 80 hydrolysis

c) Niacin test

d) Nitrate reduction test

The organism gives negative results in urease, tween 80 hydrolysis and niacin test. It is positive by nitrate reduction test.

Following example describes the process of obtaining constituents/fractions of Mycobacterium w.

The constituents/fractions of Mycobacterium w can be prepared for the purpose of invention by:

I. Cell disruption

II. Solvent extraction

III. Enzymatic extraction.

The cell disruption can be done by way of sonication or use of high pressure fractionometer or by application of osmotic pressure ingredient.

The solvent extraction can be done by any organic solvent like chloroform, ethanol, methanol, acetone, phenol, isopropyl alcohol, acetic acid, urea, hexane etc.

The enzymatic extraction can be done by enzymes which can digest cell wall/membranes. They are typically proteolytic in nature. Enzyme liticase and pronase are the preferred enzymes. For the purpose of invention cell constituents/fractions of Mycobacterium w can be used alone in place of mycobacterium w organisms or it can be added to the product containing mycobacterium w.

Addition of cell constituents results in improved efficacy of the product.

Following example describes the Method of using mycobacterium W and its constituents/fractions as an adjuvant and results of its effect on antibody levels.

To evaluate the effect of the Mycobacterium w as an adjuvant the following experiments were done.

Immunization of Horse against Rabies:

Horses immunized with rabies vaccine (Rabipur) were re-immunized by administering rabies vaccine (Rabipur) reconstituted with water or with Mycobacterium w containing normal saline. Each horse received 1.0 ml of reconstituted Rabipur intramuscularly in two doses of 0.5 ml each over each shoulder. The blood was withdrawn at the beginning (zero day) and at the

intervals of seven and fourteen days following re-immunization. The blood was analyzed for rabies antibody titer using ELISA against reference standard. The pooled blood from each group was also evaluated for neutralizing rabies antibodies using mouse neutralizing antibody test.

Figure 1 shows the effect of both vaccines on serum antibody levels in horses , three in each group, having very low initial antibody titer . The vaccine alone does not raise the antibody titer while vaccine with Mw is capable of raising the titer to significant higher level on day 7. This increases further on day 14.

Figure - 2 Shows the effect of both vaccines on serum antibody levels in horses , two in each group, having high initial antibody titer . The vaccine alone does not raise the antibody titer while vaccine with Mw is capable of raising the titer to significant higher level on day 7. This increases further on day 14.

When neutralizing antibodies were evaluated using mouse-neutralizing test, it was observed that there was no effect on neutralizing antibody levels in control group (receiving rabies vaccine alone) but there was significant improvement in a group receiving the vaccine with mycobacterium W. (Table-1)

Table-1 Mouse Neutralizing Antibody Titer against Rabies.

Group	Day 0	Day 14
Control	597 IU/ml	467 IU/ml
Mw adjuvant	468 IU/ml	1125 IU/ml

Immunization of Mice against Rabies

Mice were administered 0.1 ml of rabies vaccine (Rabipur) reconstituted in water (control) or reconstituted with Mycobacterium w (Mw) containing normal saline. In each groups thirty mice were immunized. From each group 5,10,15,20,25 and on 30th day five mice were bled to obtain sera.

The vaccine was administered 0.2 ml intradermally divided into two injections of 0.1 ml each, over each side of back. The antibody titer was evaluated subsequent to vaccine administration at the interval of 5 days. The antibody titer was measured by ELISA (Figure 3). The findings suggest that addition of Mycobacterium w achieves higher antibody tilter in comparison to control. It achieves such high titer very early. The peak value is achieved on day 10 in Mw group compared to day 15 in control group. On day 10 value achieved by Mw group is more than twice that of control group.

In a control group after reaching the peak on day 15 it declines rapidly and value achieved on day 20 is less than half of that achieved on day 15 & is not detectable on day 25 & 30. In Mw group, Value on day 20 is more than twice compared to control group, which is maintained on day 25 & day 30.

Thus Mw when used along with rabies vaccine achieves a higher peak antibody level earlier compared to control group & maintains it for prolonged period (more than 30 day).

Mouse neutralizing antibody body against Rabies.

Serum obtained on day 5 & day 10 from experiment one above was used to detect mouse neutralizing antibodies by inoculating into brain of mice along with live rabies virus.

One day 5 in both groups the neutralizing antibody titer were non detectable. On day 10 Mw group contained 1.35 IU/ml while control group contained 0.2975 IU/ml of mice neutralizing antibodies.

None of the animals demonstrated any signs of local or generalized toxicity & vaccine was well tolerated.

Above examples demonstrates adjuvant effect of Mycobacterium W to the vaccine.

Identical results are also obtained when fractions/constituents of Mycobacterium W are used.

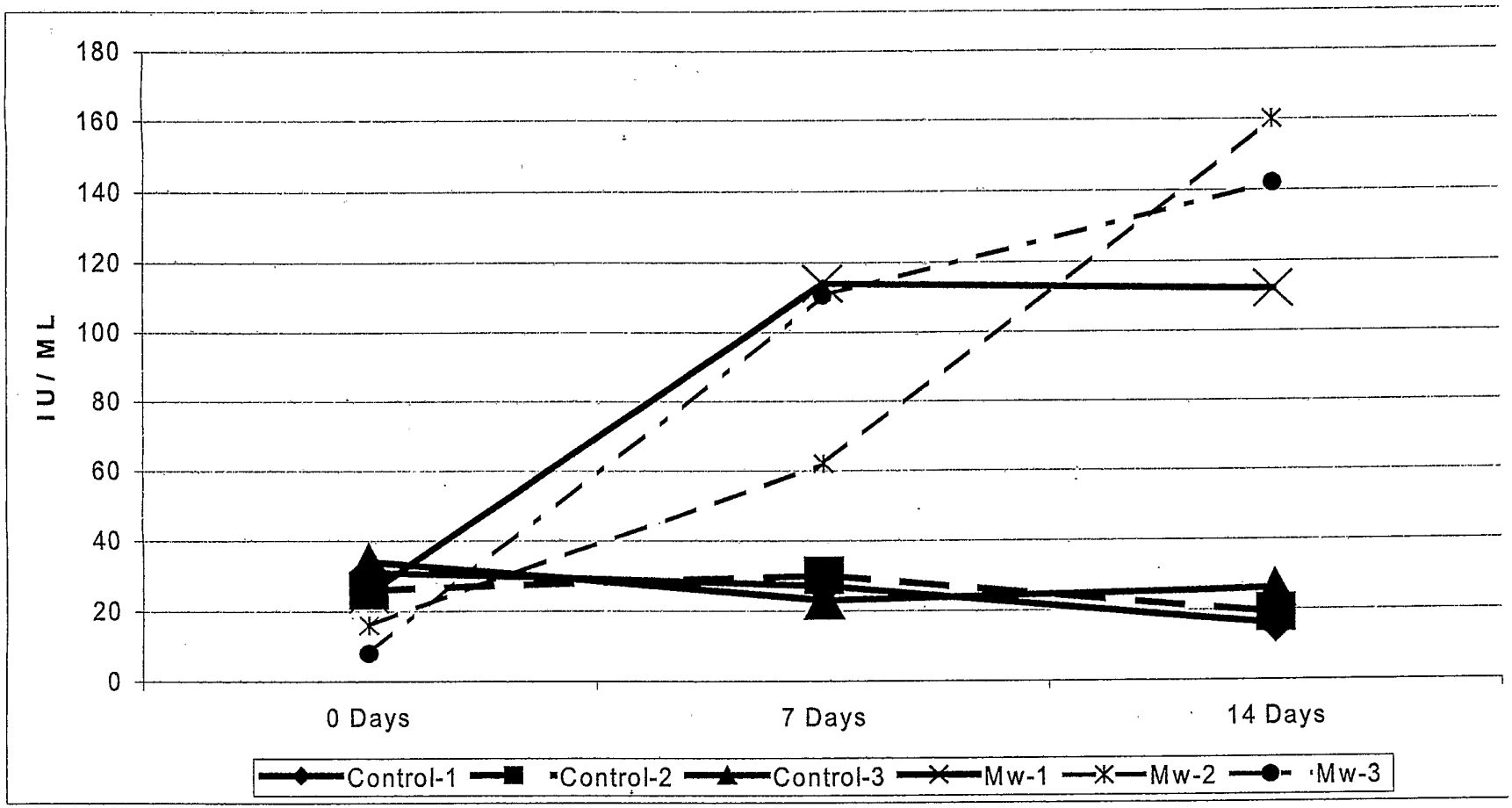

Figure - 1. Effect of adjuvant on antibodies against rabies in horses who have low titer initially.

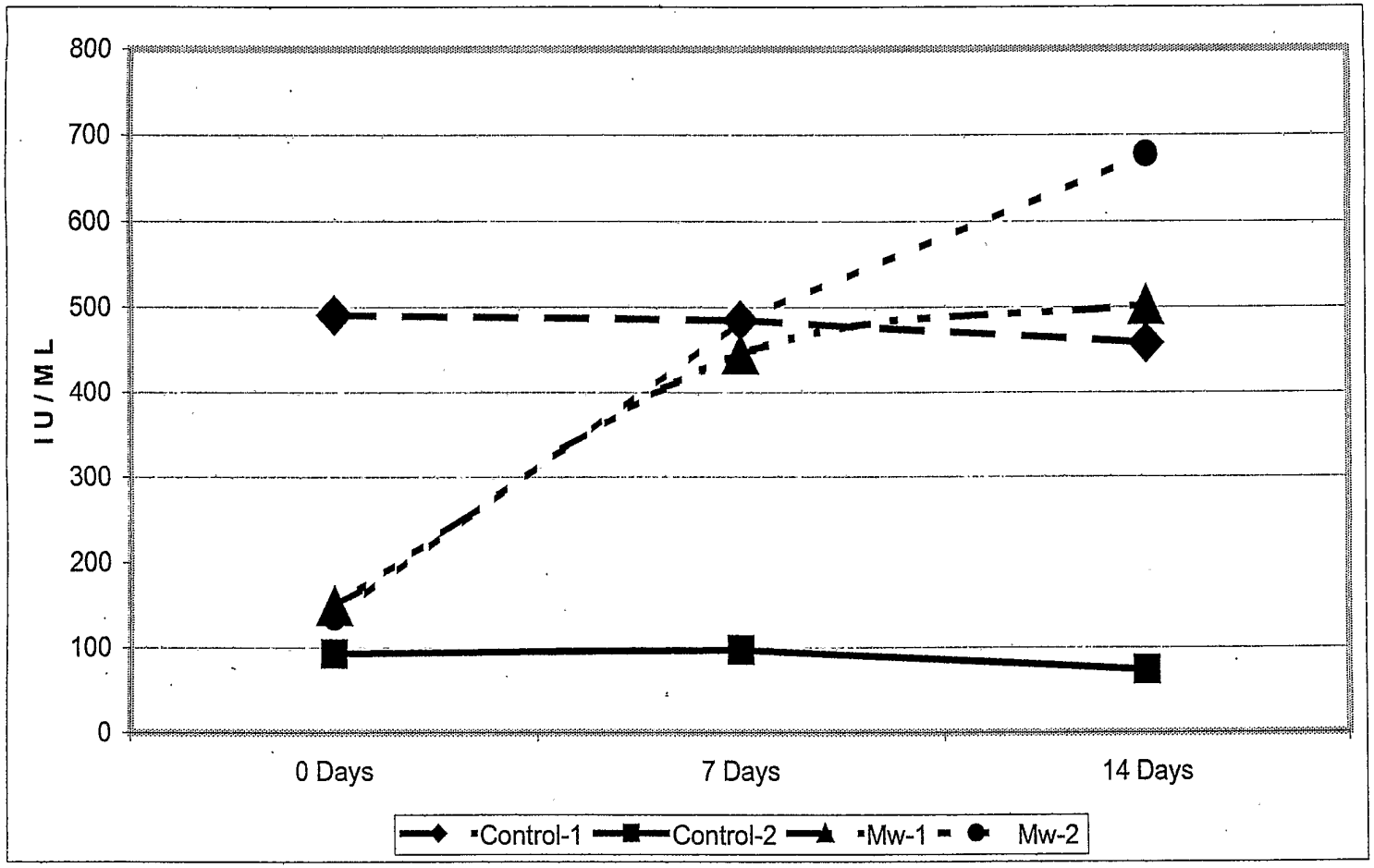

Figure-2. Effect of adjuvant on antibodies against rabies in horses who have high titer initially.

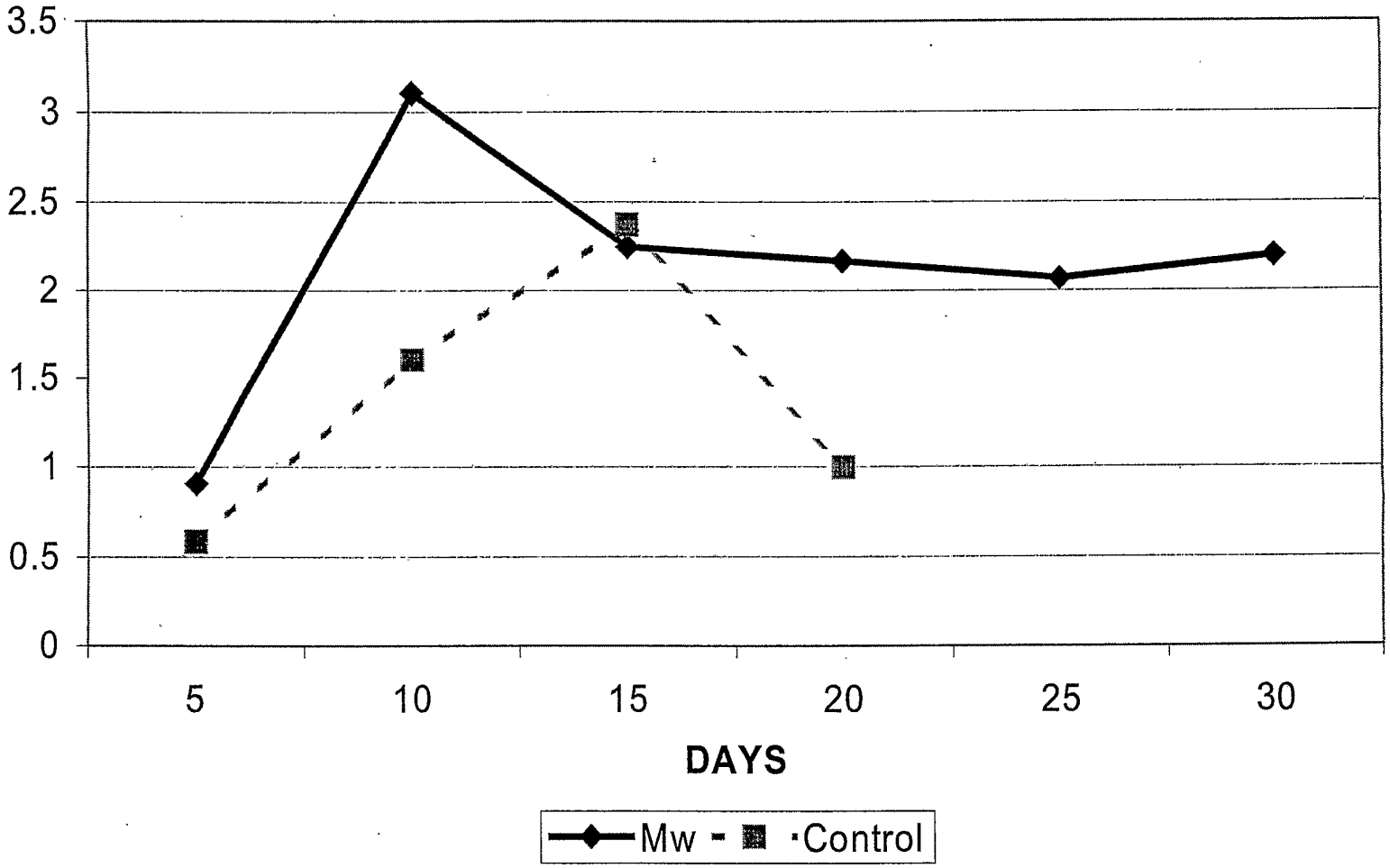

Fig.3ANTIBODY TITER FOLLOWING RABIES VACCINE IN MICE

GPA 08/0244

PCT

GENERAL POWER OF ATTORNEY

(for several international applications filed under the Patent Cooperation Treaty)

(PCT Rule 90.5)

The undersigned person(s) :
(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

Cadila Pharmaceuticals Ltd.,
"Cadila Corporate Campus", Sarkhej – Dholka Road, Bhat, Ahmedabad – 382210, Gujarat, India
Facsimile No. +91-2718-225031

hereby appoint(s) the following person as: ☐ agent ☒ common representative

Name and address
(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

KHAMAR, Balukesh Mafatlal
Cadila Pharmaceuticals Ltd.,
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to represent the undersigned before

☒ all the competent International Authorities
☐ the International Searching Authority only
☐ the International Preliminary Examining Authority only

in connection with any and all international applications filed by the undersigned with the following Office

INTERNATIONAL BUREAU OF WIPO in GENEVA as receiving Office

and to make or receive payments on behalf of the undersigned.

Signature(s) *(where there are several persons, each of them must sign; next to each signature, indicate the name of the person signing and the capacity in which the person signs. If such capacity is not obvious from reading this power)*

MODI, Indravadan Ambalal
Chairman & Managing Director

Date: March 18, 2008

Form PCT/Model of general power of attorney (for several international applications) (July 1992)